Construction of a Full-Length Murine Proα2(I) Collagen cDNA by the Polymerase Chain Reaction

Charlotte L. Phillips, Laura W. Lever, Sheldon R. Pinnell, Leigh D. Quarles, and Richard J. Wenstrup

Department of Medicine (CLP, SRP, RJW, LDQ), Division of Dermatology (CLP, SRP, RJW), Division of Nephrology (LDQ), and Department of Pediatrics (RJW, LWL), Duke University Medical Center, Durham, North Carolina, U.S.A.

Construction of large collagen cDNA has been hindered by the relatively large size and high G-C content of processed mRNA. We describe here the development of a rapid and efficient method for obtaining large full-length collagen cDNA. A full-length (4.3 kb) murine proα2(I) collagen cDNA was constructed by synthesis of a first-strand cDNA library with use of poly-A RNA (MC3T3-E1) and the oligo-dT_{17}-adapter primer described by Frohman et al (Proc Natl Acad Sci USA 85:8998, 1988). Proα2(I) collagen cDNA were specifically amplified by the polymerase chain reaction (PCR) with a proα2(I) specific primer as the 5′ primer (20mer; corresponding to nucleotide positions 42–61 in the first exon of the murine proα2(I) collagen gene, COL1A2), and with the adapter sequence 5′ to the dT_{17} as the 3′ primer. The PCR conditions were optimized to allow amplification of the expected 4.0–5.0-kb product; a major 4.3-kb product was visualized by ethidium bromide, identified by in situ gel hybridization, and cloned. DNA sequencing determined that it contained the correct 5′ sequence and the 3′ end had a 68 basepair (bp) 3′ untranslated region. The entire sequence that codes the amino-terminal propeptide domain has been determined and compared to the human sequence. The homology between human and mouse is less in the amino terminal propeptide than in the triple helical domain; exon 5 of murine COL1A2 codes for an additional six amino acids not found in human COL1A2. *J Invest Dermatol 97:980–984, 1991*

At present, the family of collagens consists of 14 types, which are coded for by a minimum of 25 genes (reviewed in [1–3]). The fibril-forming collagens, which include types I, II, III, V, and XI, have long uninterrupted triple helices composed of three α chains with a primary structure of $(Gly\text{-}X\text{-}Y)_n$, where X and Y are frequently hydroxyproline and proline. The fibrillar collagens and their genes have been relatively well characterized; mutations in proα1(I), proα2(I), proα1(II), and proα1(III) collagen genes (COL1A1, COL1A2, COL2A1, and COL3A1, respectively) have been associated with several skeletal dysplasias [1–7]. To develop models of heritable connective tissue disease and to examine the function of collagen in tissue, it is important to be able to express fully functioning fibrillar collagen chains. However, the construction of full-length collagen expression genes for these experiments is made more difficult by several factors that are distinctive to fibrillar collagens. In addition to the relatively large size (4–6 kb) of the processed mRNA, the fibrillar collagen mRNA and cDNA are very G-C rich, because the triple helical domain is 33% glycine (codon: GGN) and 20–30% proline (codon: CCN) [2]. This also contributes to the multiple homologous internal sequences throughout the triple helical domain that can make it a challenge to identify full-length fibrillar collagen cDNA by standard colony hybridization techniques.

We isolated a full-length murine proα2(I) collagen cDNA using the polymerase chain reaction (PCR) in a rapid and efficient strategy that may be useful for cloning other full-length fibrillar collagen cDNA. Described in the following report is the optimization of this procedure, and the amplification and cloning of a 4.3-kb murine proα2(I) collagen cDNA. DNA sequences that code the amino-terminal propeptide and portions of the triple helical domain of the murine proα2(I) chain cDNA have been sequenced and compared to the human proα2(I) sequence.

MATERIALS AND METHODS

Probes and Primers The hybridization probe used in Northern blot analyses was a 1375-bp human proα2(I) cDNA corresponding to nucleotide position 1429–2804 [8]. For in situ gel hybridizations, a 30-bp oligonucleotide, h1301, complimentary to nucleotide position 1301–1330 of human proα2(I) cDNA, was used. The first-strand cDNA synthesis primer was the oligo dT_{17}-adapter primer described by Frohman et al, a 35mer with the sequence 5′GACTCGAGTCGACATCGAT_{17}′3 [9]. The 3′ PCR primer was the 18mer containing the adapter primer sequence 5′ to dT_{17}. The 5′ PCR primers were 20mers, either m42 or m181, which

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Reprint requests to: Dr. Charlotte L. Phillips, Duke University Medical Center, Post Office Box 3135, Durham, NC 27710.

Abbreviations:
bp: base pair
DMEM: Dulbecco's modified Eagle's medium
dNTP: deoxynucleotide
DTT: dithiothreitol
EDTA: ethylene diamine tetraacetate
NaPP: sodium pyrophosphate
PCR: polymerase chain reaction
SDS: sodium dodecyl sulfate
UV: ultraviolet

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corresponded to nucleotide positions 42–61 and 181–200 in the first exon of the mouse proα2(I) collagen gene, respectively; nucleotide position 1 is the start of transcription [10].

Northern Blot Hybridization Total RNA (5 μg) isolated from human newborn dermal fibroblasts and MC3T3-E1 cells [11] was fractionated by size using formaldehyde gel electrophoresis [12], transferred to GeneScreen Plus membrane (New England Nuclear, Boston, MA) and hybridized according to the manufacturer's recommended conditions. The proα2(I) collagen cDNA was labeled with [α^{32}P]dCTP using random primer extension [13].

First-Strand cDNA Synthesis and Polymerase Chain Reaction Total RNA was isolated from the murine osteoblast cell line, MC3T3-E1 (derived from newborn calvaria) [14], which had been seeded at a density of 20,000 cells/100 mm diameter culture dish (Falcon) and grown for 25 d in F/12 Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 50 μg/ml ascorbate, and 10 mM β-glycerol phosphate. Poly-A RNA was isolated by oligo-dT chromatography and first-strand cDNA synthesis was performed as described by Gubler and Hoffman [15] in a 100-μl reaction volume with 5 μg poly-A RNA and 625 ng of oligo dT_{17}-adapter primer [9]. Amplification of full-length murine proα2(I) cDNA was performed as follows except where otherwise indicated, in a 100 μl reaction containing 20 ng cDNA, 60 ng of each of the 5′ and 3′ amplification primers, 200 μM of each deoxynucleotide (dNTP), 1.5 mM Mg^{++}, 10 mM dithiothreitol (DTT), 50 mM KCl, 10 mM tris-HCl, pH 8.3, 0.01% gelatin and Taq polymerase, 2.5 units. All the reactions were run under the following protocol for 40 cycles with denaturation at 94°C for 1 min, 10 seconds, annealing at 54°C for 2 min, 10 seconds, and extension at 72°C for 6 min in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). After each cycle, 10 additional seconds were added to the extension step.

At completion of the PCR run, 15 μl of each reaction mixture was analyzed by agarose gel electrophoresis using a 1% agarose gel, 1 × TAE buffer (40 mM tris-acetate, 1 mM ethylene diamine tetraacetate [EDTA]) with 50 ng/ml ethidium bromide (EtBr), and the amplification products were visualized under ultraviolet (UV) lamp and photographed. These gels were then hybridized in situ.

In Situ Gel Hybridization The gels were denatured at 4°C, 30 min, in 0.5 N NaOH, 1.5 M NaCl, then neutralized at 4°C, 30 min, in 1 M tris, pH 7.4, 1.5 M NaCl, and dried under vacuum at 60°C. The gels were rehydrated in deionized H_2O, pre-hybridized for 1 h at 42°C in 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0), 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml sheared and denatured salmon sperm DNA, 0.05% sodium pyrophosphate (NaPP), and then hybridized overnight at 42°C in 6 × SSC, 1 × Denhardt's solution, 20 μg/ml tRNA, 0.05% NaPP, with 1 × 10^6 cpm/ml of P^{32} 5′end-labeled α2(I) oligonucleotide probe, h1301. The gel was then washed twice with 6 × SSC, 0.05% NaPP at room temperature, 5 min, followed by a single 1-h wash at 42°C, and then exposed to x-ray film.

Amplification and Cloning of the Murine Proα2(I) Collagen cDNA Murine proα2(I) collagen first-strand cDNA was amplified by PCR according to the protocol described above using m42 as the 5′ primer. The PCR products were separated according to size by agarose gel electrophoresis, the 4.0–5.0-kb products excised from the gel and purified using the Geneclean system (Bio 101, Inc., La Jolla, CA). These products were digested with *Sph I* (restriction site at position +54 of murine proα2(I) collagen gene) and *Sal I* (restriction sites in the adapter sequence and at the equivalent nucleotide position 1456 in human proα2(I) cDNA) [8]. The resulting two murine proα2(I) collagen cDNA fragments were purified by gel electrophoresis and Geneclean; followed by ligation into pBS vectors (Stratagene, La Jolla, CA), which had been previously digested with *Sal I* alone, or *Sph I* and *Sal I* together and phosphatased with bovine alkaline phosphatase (Gibco-BRL, Gaithersburg, MD).

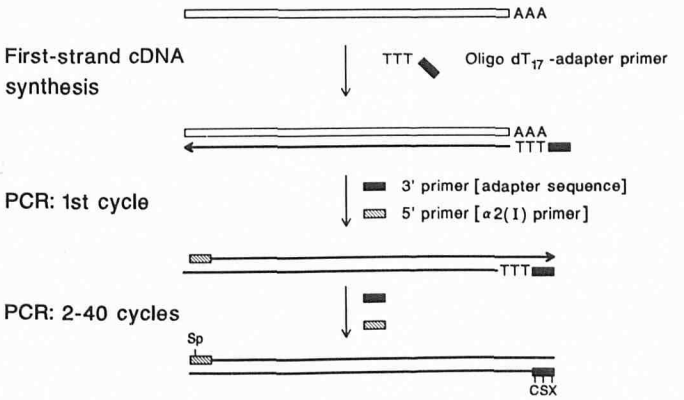

Figure 1. Strategy for synthesis and amplification of the murine proα2(I) collagen cDNA using PCR. The amplification product contains at its 5′ end a *Sph I* (Sp) restriction site and at its 3′ end the *Cla I* (C), *Sal I* (S), and *Xho I* (X) restriction sites to aid cloning.

These were transformed into the *Eschericha coli* strain DH5α (Gibco-BRL) and sequenced using the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's recommendations. To construct the full-length murine proα2(I) collagen cDNA the 5′ fragment was digested with *Sph I* and *Sal I*, and ligated to the 3′ fragment that had been digested with *Sal I* and *Cla I*; this *Sph I-Cla I* ligated product was purified by gel electrophoresis and then ligated into pSP72 (Promega Corporation, Madison, WI), which had been previously digested with *Sph I* and *Cla I*, followed by a phosphatase reaction. DH5α cells (Gibco-BRL) were then transformed by this ligation reaction and several clones identified and sequenced.

RESULTS AND DISCUSSION

Amplification and Cloning Strategy of Murine Proα2(I) Collagen cDNA To specifically and efficiently synthesize a full-length murine proα2(I) collagen cDNA, we developed a strategy that utilized a procedure by Gubler and Hoffman, which promotes the synthesis of full-length cDNA [15], in conjunction with a procedure decribed by Frohman et al for the amplification of specific cDNA by the polymerase chain reaction [9] (Fig 1). To amplify selectively and clone full-length murine proα2(I) collagen cDNA, we employed poly A RNA isolated from MC3T3-E1 cells to synthesize a library of first-strand cDNA using an oligo dT_{17}-adapter primer. This primer has two advantages over the traditional oligo dT primer; the adapter sequence 5′ to the oligo dT_{17} serves as a better template for the PCR than poly dT and it contains several possible cloning sites (*Cla I*, *Sal I*, and *Xho I*).

In human dermal fibroblasts, between 3 and 5 mRNA species (4.3–5.1 kb) of the proα2(I) gene, COL1A2, are transcribed. These transcripts are colinear; the 3′ untranslated sequences are of variable lengths [8,16]. The larger transcripts utilize polyadenylation signals that are further downstream than those utilized by the smaller transcripts. Northern blot hybridization of total RNA from human newborn dermal fibroblast and murine osteoblast (MC3T3-E1) cell lines to a human proα2(I) collagen cDNA demonstrated that MC3T3-E1 cells synthesize homologous proα2(I) mRNA species of similar sizes as human dermal fibroblasts (Fig 2). Thus, the size of full-length murine proα2(I) collagen cDNA were expected to be between 4.0 and 5.0 kb. The DNA sequence from the first exon of murine proα2(I) gene (COL1A2) has been previously reported [10]; this sequence allowed the use of the polymerase chain reaction to amplify full-length proα2(I) cDNA. Two oligonucleotides, either m42 or m181, corresponding to nucleotide positions 42–61 or to 181–200 in the first exon of the murine proα2(I) gene, respectively, were used as 5′ amplification primers. Primer m181 was used in determining the optimum PCR conditions. Primer m42 was used

Figure 2. Murine proα2(I) mRNA are of similar size and are homologous to human proα2(I) mRNA. Five micrograms of total RNA from human dermal fibroblasts (1) and MC3T3-E1 cells (2) were separated by gel electrophoresis, transferred to GeneScreen Plus membrane, and hybridized to a human proα2(I) cDNA (see *Materials and Methods*). Sizes of the human RNA transcripts, which were determined by sequence analysis, are indicated [8].

to take advantage of the *Sph I* site at nucleotide position +54 to aid in cloning.

Effects of Template, dNTP, and Mg^{++} Concentrations on the PCR To determine the most efficient conditions necessary to specifically amplify the 4.0–5.0-kb murine proα2(I) collagen cDNA, we examined the effects of Mg^{++}, dNTP, and first-strand cDNA template concentrations. Figure 3*A* presents the results from the examination of the effects of different concentrations of Mg^{++}

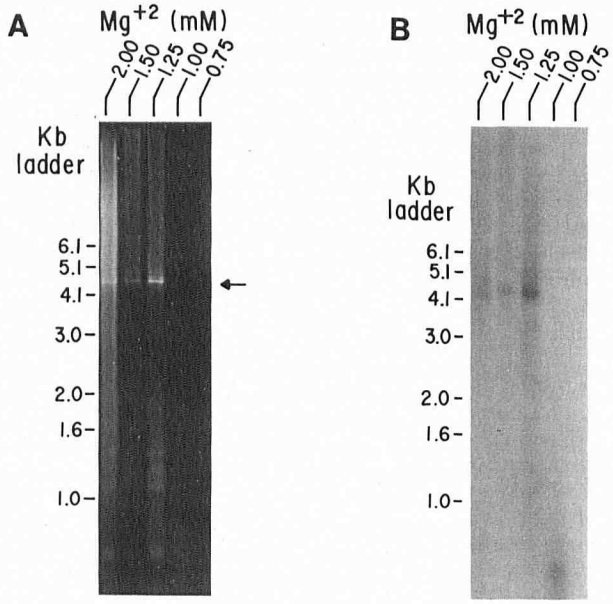

Figure 3. Effects of Mg^{++} concentrations on amplification efficiency of murine proα2(I) cDNA. *A,* The PCR products from reactions with the indicated Mg^{++} concentrations were separated by agarose gel electrophoresis and visualized with ethidium bromide. *Arrow,* the primary amplification product. *B,* In situ hybridization to this same gel using a human α2(I) oligonucleotide probe, h1301 (see *Materials and Methods*).

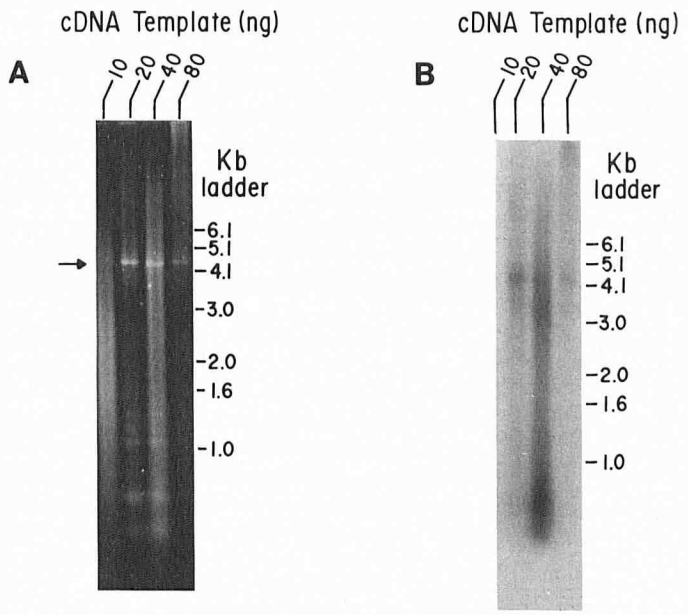

Figure 4. Effects of cDNA template concentration on amplification efficiency of murine proα2(I) cDNA. *A,* The PCR products from reactions with the indicated cDNA template concentrations were separated by agarose gel electrophoresis and visualized with ethidium bromide. *Arrow,* the primary amplification product. *B,* In situ hybridization to this same gel using a human α2(I) oligonucleotide probe, h1301 (see *Materials and Methods*).

on the amplification of murine proα2(I) cDNA. PCR was performed as described in *Materials and Methods* except that Mg^{++} was at the following concentrations: 2 mM, 1.50 mM, 1.25 mM, 1 mM, and 0.75 mM. The reaction products were fractionated by size by agarose gel electrophoresis and the DNA visualized with ethidium bromide (Fig 3*A*). These results show that not only is there a narrow range of Mg^{++} concentrations in which amplification of the 4.0–5.0-kb products can occur but also that there was amplification of at least three large DNA species; a major 4.3-kb species and two slightly larger minor species. The relative amounts of these three species appear consistent with the Northern blot hybridization results (Fig 2). To determine if these amplification products were proα2(I) collagen cDNA, we dried the agarose gel under vacuum, and performed in situ hybridization using the oligonucleotide probe h1301, (human proα2(I) collagen). Figure 3*B* shows that h1301 hybridizes to the amplification products. These results are consistent with the hypothesis that the different mRNA species of murine proα2(I) collagen RNA are due to different length 3′ untranslated regions and not to different transcriptional start sites [8,16].

The concentration of the cDNA template was determined to be most effective in the PCR when it was greater than 10 ng; however, when it was more than 40 ng, the efficiency of amplification decreased (Fig 4). Different dNTP concentrations from 100 mM to 400 mM were found to have little effect on the amplification efficiency at a Mg^{++} concentration of 1.5 mM (data not shown). There is some suggestion that dNTP concentrations of 400 mM and greater may increase the error rate of Taq polymerase [17]. The relative concentrations of Mg^{++} and dNTP to each other have been reported to have dramatic effects on amplification efficiencies. In this instance, the concentration of Mg^{++} appeared to be the most dramatic effector of amplification efficiency. The PCR was optimum with 1.25 mM Mg^{++} and 200 mM dNTP, and there was virtually no amplification at 1 mM Mg^{++} and 200 mM dNTP (Fig 3*A*, 3*B*).

Cloning and Characterization of the Murine Proα2(I) Collagen cDNA Following the PCR, which had used m42 as the 5′ primer in order to synthesize a full-length murine proα2(I) cDNA,

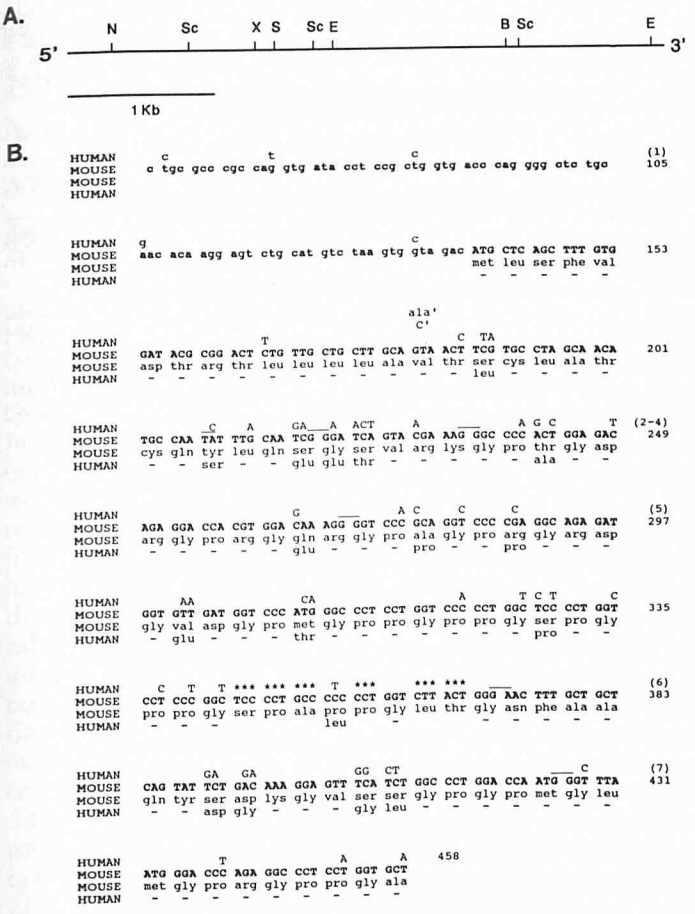

Figure 5. *A,* A partial restriction map of the murine proα2(I) collagen cDNA. The restriction sites for the enzymes *Nco I* (N), *Sac I* (Sc), *Xho I* (X), *Sal I* (S), *Eco RI* (E), and *Bgl II* (B) are indicated. The enzymes *Bam HI, Cla I, Hind III, Eco RV,* and *Sph I* did not cut. The *Sph I* site was altered and lost during cloning. *B,* Comparison of the nucleotide and amino acid sequences of the amino propeptide of murine and human proα2(I). *Capital letters,* coding region: *lower case letters,* untranslated region. Nucleotides are numbered from 60 (where 1 is the start of transcription according to Rossi et al [10]). *Top line,* nucleotide sequence of human amino-terminal propeptide cDNA [8,16] where it differs from the murine sequence. *Lines 2* and *3* are the nucleotide and amino acid sequences of the murine amino-terminal propeptide. *Line 4* is the amino acid sequence coded by human. *Dashes,* identical residues; *asterisks,* missing nucleotides or amino acids. The (') indicates the differences between this murine proα2(I) collagen and the previously published partial sequence of the murine proα2(I) collagen gene [20]. The boundaries of the human proα2(I) exons are signified by the lines over the corresponding mouse sequences. The exon number (in parenthesis) and nucleotide numbering are shown on the right.

the amplification products were digested with the restriction enzymes *Sph I* and *Sal I. Sph I* was known to cut the murine proα2(I) collagen gene at position +54 in the 5′ untranslated region.

Predictions based on human proα2(I) cDNA sequence suggested that there would not be any additional *Sph I* or *Sal I* sites other than the *Sal I* site in the adapter sequence. The adapter sequence contained less common restriction sites (*Cla I, Sal I,* and *Xho I*) for cloning. However, there was an unexpected *Sal I* site at the murine equivalent of nucleotide position +1452 of human proα2(I) cDNA [8]. The 5′-*Sph I-Sal I* fragment and the 3′-*Sal I-Sal I* fragment were cloned and sequenced independently to verify their identity and orientation. To construct the full-length murine proα2(I) collagen cDNA, we ligated the 5′-*Sph I-Sal I* fragment to the 3′ fragment, which had been digested with both *Sal I* and *Cla I;* there is a *Cla I* site 5′ to the *Sal I* site in the adapter sequence (Fig 1). The resulting product, the full-length murine proα2(I) collagen cDNA *(Sph I-Cla I),* was then ligated into pSP72 vector that had been previously digested with *Sph I* and *Cla I.*

DNA mapping and sequence analysis of several clones determined that the murine proα2(I) collagen cDNA were 4.3 kb in length, beginning at position +59 after the transcription start site, and they had a 68-bp 3′ untranslated region. Figure 5*A* is a partial restriction map of the murine proα2(I) cDNA. Preliminary sequence analysis of the triple helical domain indicates that there is a high degree of homology between human and mouse proα2(I) sequences; all the residue differences are at the X and Y positions and are conservative substitutions for hydrophobic residues (data not shown) [8,19]. The amino-propeptide and 5′ end of the triple helix (exons 1–7) were sequenced in their entirety and compared to the partial mouse proα2(I) sequence previously published by Schmidt et al [20] and to the human proα2(I) sequences (Fig 5*B*) [8,19]. There was a single nucleotide difference between this mouse proα2(I) sequence and the partial mouse sequence previously published: nucleotide 182 is T rather than C, which codes for a valine rather than an alanine [20]. This is a conservative difference and could represent a polymorphism or interspecies variation. A valine at this position is present in both human and chick proα2(I) collagen. There is less homology in this region between human and mouse than in the triple helical domain. Based on the reported exon boundaries of human COL1A2 [8,19] within exon 5 of the murine COL1A2 there are an additional 18 bp that code for six more amino acid residues than human COL1A2. This variability is not totally unexpected; exon 5 of chick COL1A2 codes for three less amino acid residues than human and nine less than mouse [19].

The procedure described here offers a rapid and efficient protocol for obtaining long, full-length fibrillar collagen cDNA, requiring only limited amounts of sequence information. This procedure may prove useful not only for synthesis of other full-length fibrillar collagen cDNA, but also for construction of collagen expression systems for analyses of collagen structure–function relationships in cell culture and animal experiments.

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ANNOUNCEMENT

The Alopecia Areata Research Foundation is a non-profit organization that was started in 1983 for the sole purpose of supporting research into the causes and treatment of alopecia areata. This year, the Alopecia Areata Foundation is pleased to announce the availability of a $10,000.00 grant to fund a research project related to alopecia areata.

This research grant may be used to support either bench research or clinical research in the fields of dermatology or immunology as related to alopecia areata. Potential applicants are urged to submit their protocols and curriculum vitae to the Alopecia Areata Foundation. The protocols should not exceed three pages (including references) and should include background, purpose, methodology, and methods of analysis. The deadline for applications is March 1, 1992 with funding available as early as April 1, 1992. The applications will be reviewed by the Alopecia Areata Research Foundation's Medical Advisory Board.

For further information or questions regarding grant proposals, please contact Mr. Whitfield Lee, Executive Director, Alopecia Areata Research Foundation, P.O. Box 17264, Raleigh, North Carolina, 27619.